Running head: EFFECTS OF ESTROGEN ON AGGRESSIVE BEHAVIOR

Effects of Estrogen on Aggressive Behavior

A Senior Honors Thesis

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by

Kelly Greiwe

The Ohio State University

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Project Advisor: Professor Randy Nelson, Department of Psychology

Abstract

Estrogens are known modulators of aggression however, the specific action of estrogens on brain function has not been determined. In a correlational study, the number of estrogen receptors in regions of the hypothalamus and limbic system was correlated with aggressive behavior in a domestic strain of mice (CD-1). To determine whether these correlations reflect the effect of estrogen on aggression, the production of estrogen was manipulated by an aromatase inhibitor (fadrozole) to observe the subsequent effects on aggressive behavior. Reduction of estrogen production resulted in a decrease in aggressive behavior, suggesting that estrogen acts to increase aggression.

Effects of Estrogen on Aggressive Behavior

Previous studies have shown that estrogens increase the probability of aggressive behavior in domestic mice. For example, treatment of castrated mice with estradiol increases this probability in two strains of domestic mice (*Mus musculus*; CFW and CF-1 strains) as compared to mice treated with oil (Simon and Whalen, 1986). However, these studies have addressed the likelihood of aggressive behavior and not the intensity. Moreover, there is contradictory evidence in other species of mice. For some species, it seems that estrogen may decrease the expression of aggressive behavior. For example, blocking the production of estrogen increased aggressive behavior of California mice relative to control animals (Trainor et al., 2004). For this study, the mice were housed in long days, and the effect of estrogen in this genus has been shown to depend on photoperiod. Estrogen increases aggression in short, winter-like days, whereas estrogen decreases aggression in long, summer-like days (Trainor & Nelson, unpublished). It appears that many variables may influence the effect of estrogen on aggressive behavior, including the expression of estrogen receptor subtypes.

Two estrogen receptor subtypes are known to exist: the α estrogen receptor (White et al., 1987) and the β estrogen receptor (Kuiper et al., 1996). The different effects of these two subtypes have been studied primarily with knock-out mice. A knock-out mouse is a mouse in which a gene of interest has been selectively inactivated. This gene is inactivated prior to birth and remains ineffective throughout development. Estrogen receptor α knock-out (ERαKO) mice were less aggressive than wild-type, or genetically intact, normal, mice (Ogawa et al., 1998). Conversely, sexually experienced adult estrogen receptor β knock-out (ERβKO) mice exhibit increased aggressive behavior as compared to wild-type mice (Ogawa et al., 1999). However, this trend toward increased aggression does not hold with sexually inexperienced ERβKO mice.

The sexually inexperienced ERβKO mice are more aggressive as juveniles but not as adults (Nomura et al., 2002). Sexual experience was shown to possibly modify the effect of estrogen receptor beta on aggression, highlighting that other social behaviors may influence aggressive behavior. How exactly these receptor types modulate behavior is uncertain.

It has been suggested by knock-out studies that estrogen receptor β has an inhibitory effect on the transcription of estrogen receptor α (Weihua et al., 2000; Liu et al., 2002). This follows directly from the observation that lacking estrogen receptor β increases aggressive behavior, and therefore, the receptor must act to moderate the effects of estrogen receptor α in some way. However, knock-out studies cannot differentiate between developmental effects and effects on the adult mouse. There are also many studies suggesting that the two subtypes affect different motivational systems. Understanding how estrogen is created and functions in the brain is extremely important in grasping the mechanism by which it affects behavior.

Aromatase is an enzyme that converts androgens to estrogens. The loss of this enzyme results in the cessation of estrogen production. Consistent with this reasoning, aromatase knock-out mice have been found to be less aggressive in resident-intruder tests than wild-type mice (Toda et al, 2001). Again, this suggests that estrogen plays an important role in aggressive behavior, but also suggests that aromatization can regulate aggressive behavior in domestic mice. However, it remains unclear where exactly aromatase acts in the brain. This link is vital to discovering the mechanism by which estrogen influences aggressive behavior.

Many different brain regions have been implicated in the study of aggressive behavior. Specifically, in female California mice c-fos expression was greater in mice tested in a resident-intruder aggression test than control mice for a variety of brain regions (Davis ES, Marler CA, 2004). C-fos expression, which is quantified using immunocytochemistry, is a measure of brain

activation. Areas of the limbic system and hypothalamus have been considered particularly important in the expression of aggression. Furthermore, Davis and Marler (2004) found that c-fos expression increased in the bed nucleus of the stria terminalis (BNST) and ventral lateral septum (vLS) only in the more aggressive females, suggesting that these areas may be of particular interest. Although these brain regions are believed to be important in the regulation of aggressive behavior, various regions of the limbic system have been implicated in a wide array of social behaviors.

A "social behavior circuit" (Newman, 1999) has been proposed involving six areas of the limbic system: the BNST, lateral septum (LS), midbrain, ventral medial hypothalamus (VMH), anterior hypothalamus (AH), and medial pre-optic area (MPOA). These areas are believed to be part of a neural circuitry modulating several social behavior systems. The six regions are activated to different degrees for these different behavioral systems. Hypothetical representations of the c-fos activation can be used to compare the relative activation levels of the six regions in various social behaviors. In male domestic mice, the BNST, LS, and VMH are strongly activated in aggressive behavior. These areas were therefore the primary areas of interest for the present study as they have been deemed the most likely regions in which a relationship between estrogen and aggressive behavior may be found.

In the first study, correlational relationships between estrogen receptors in regions of the hypothalamus and limbic system and aggressive behavior in CD-1 domestic mice were examined. The data from this study suggested that estrogen may act to increases aggressive behavior in this species. In order to establish a direct relationship between estrogen expression and aggressive behavior, estrogen production was then manipulated by fadrozole, an aromatase inhibitor. The production of estrogen manipulated through this treatment functioned as the

independent variable. The dependent variable was aggressive behavior. Aggressive behavior was quantified by aggressive variables measured in the resident-intruder aggression test, specifically attack latency, time spent boxing, and number of bites. It was hypothesized that eliminating the production of estrogen would decrease aggressive behavior, as measured by the resident-intruder aggression test.

Method

Subjects

For the correlational experiment, male CD-1 mice were provided with water and Harlan Teklad 8640 *ad libitum.* For the hormone manipulation experiment, male CD-1 mice from Charles River were individually housed upon arrival, provided with water and Harlan Teklad 2016 (phytoestrogen free) *ad libitum,* and operated on at approximately 8 weeks of age. CD-1 mice are outbred and therefore genetically diverse. These individual differences are necessary to obtain valid correlations.

Design

The relationship between ERα immunopositive cells and aggressive behavior was analyzed using Pearson's correlations. For the hormone manipulation experiment, a non-parametric Kruskal-Wallis test was used followed by pair-wise Mann-Whitney U tests.

Correlational Experimental Procedure

After being housed for 8 weeks, the subjects were tested in resident-intruder aggression tests. In this paradigm, the male resident should act aggressively in defense of his territory. The subject mouse acted as the resident, and an unfamiliar male, the intruder, was placed into the resident's cage. The interactions of the mice were then videotaped for 10 minutes. Aggressive behavior variables were then scored from the videos using the Observer program. These variables include attack latency, time spent boxing, and number of bites. Forty-five minutes following the aggression test, the subject mouse was anesthetized with isoflurane, the mouse was decapitated, and its brain was removed and fixed in a five percent acrolein in phosphate-buffered saline (PBS) solution at 4°C overnight. The brain was then transferred to a 30% sucrose solution for 24 hours, and then frozen on dry ice and stored at -80°C.

Each brain was sectioned into 40 micrometer sections on a cryostat and each section was stored in 0.5 mL of PBS. Starting six sections before the crossing of the anterior commissure, every other brain section was transferred into a screened tray for ERα immunocytochemistry. There were five to six sections per well in the screened tray, and the tray was filled with 20 mL of PBS. The sections were washed three times in PBS for five minutes each time. A sodium borohydride solution was prepared with 74 mg of sodium borohydride in 20 mL of PBS. The PBS solution was blotted dry from the tray and the screened tray was transferred into a new tray containing the sodium borohydride solution for a period of 10 minutes. At the end of the 10 minutes the tray was transferred to a new tray containing the blocking solution. These two solutions act to prevent background staining.

The blocking solution was made with 14 mL of PBS, four mL of normal goat serum, and two mL of three percent hydrogen peroxide. The tray was gyrated in this solution on a shaker for 20 minutes. While the sections were in this solution, the primary ERα antibody was prepared. It was prepared by adding 1.5 uL of rabbit ERα antibody to 24.5 mL of PBS-TritonX and 500 uL of normal goat serum. One mL of the primary antibody solution was pipetted into each well of a 24 well plate. The sections were then transferred to these wells and the tray was placed on a shaker in the refrigerator for 48 hours. The primary antibody attaches to the protein of interest in this case, ERα.

At the end of the 48 hours, the sections were transferred back into a screened tray and washed in PBS three times for five minutes each time. Washing the sections in PBS will clear the unattached primary antibody so that only the antibody attached to the ERα protein remains. A secondary antibody composed of anti-goat antibody was then poured into a new tray and the screen was transferred into this tray for 90 minutes. This secondary antibody attaches to the

primary antibody. Again the sections were washed three times in PBS to wash off the excess secondary antibody. Next, the ABC solution was prepared (Vector Laboratories). This solution contains a biotin complex conjugated to horseradish peroxidase and is used to amplify the signal from the secondary antibodies. It is prepared by adding 6 drops of A and 6 drops of B to 20 mL of PBS. The screened tray was transferred into this ABC solution for 30 minutes. At the end of this time the sections were washed three times in PBS and were ready to be developed with DAB.

The diaminobenzidine, or DAB, was prepared by adding 6 drops of buffer solution, 12 drops of diaminobenzidine, 6 drops of hydrogen peroxide and 6 drops of a nickel solution to 15 mL of PBS. Staining of the sections occurred when the screened tray was placed in the DAB tray for 1.5 minutes of development. After development the sections were washed three times in PBS to prevent further development resulting in extremely dark staining. The stained sections were then mounted on gelatin-coated slides. The sections were allowed to dry and then were dehydrated with a procedure using ethyl alcohol and xylene. The slides were placed in a slide boat and dipped into 95 percent ethanol for one minute, and then a series of three containers of 100 percent ethanol for one minute each, and finally three containers of xylene for one minute each. Upon removal from the xylene, the slides were cover slipped in order to be able to examine them under a microscope without damaging the brain sections.

Images of the areas of interest including the bed nucleus of the stria terminalis, the lateral septum, and the anterior hypothalamus were identified using the mouse atlas by Franklin and Paxinos (1997) and then captured using a Nikon E800 microscope. Using a grid, a smaller region from the image of each brain area was established as the region to be counted based on

landmarks in the brain. Then the stained cells of each brain region of each subject mouse were counted with the Neurolucida software to quantify the ERα cells.

Hormone Manipulation Experimental Procedure

The mice were randomly divided into four groups: (1) empty implant group, (2) control group, (3) fadrozole group, and (4) intact group. The empty implant group was castrated, given an empty implant, and given an osmotic minipump of saline. The control group was castrated and given a testosterone implant and an osmotic minipump of saline. The fadrozole group was castrated and given a testosterone implant and an osmotic minipump of fadrozole. The intact group received sham surgery in which the testes were not removed. After these surgeries the animals were allowed 12 days to recuperate. On the thirteenth day, they were tested in a resident-intruder aggression test described above.

Results

Relationships between ERα Immunoreactivity and Aggressive Behavior

For several areas of the hypothalamus and limbic system, ERα-ir cells were consistently correlated with aggressive behavior. The total number of bites over the 10 minute resident-intruder test was positively correlated with ERα-ir in the BNST ($r = 0.67$, $p = 0.02$), LS ($r = 0.67$, $p = 0.02$), and AH ($r = 0.59$, $p = 0.04$). Aggressive behavior (as measured by number of bites) was not correlated with ERα-ir cells in the VMH ($r = -0.14$, $p = 0.67$) or MPOA ($r = .16$, $p = 0.64$).

Effect of Hormone Manipulation on Aggressive Behavior

The Kruskal-Wallis test revealed significant effects of the hormone manipulation on tailing rattling ($\chi_3 = 8.66$, $p = 0.034$) and biting ($\chi_3 = 9.37$, $p = 0.025$), but not on boxing ($\chi_3 = 5.90$, $p = 0.117$). Pair-wise comparisons were then made with Mann-Whitney U tests. The fadrozole

group displayed significantly fewer bites than the intact group ($U = 34.00$, $p = 0.037$), the empty implant group ($U = 41.50$, $p = 0.008$), and the control group ($U = 31.50$, $p = 0.045$). The fadrozole group also exhibited significantly less tail rattling than the empty implant group ($U = 41.5$, $p = 0.006$) and significantly fewer bouts of boxing than the control group ($U = 26.5$, $p = 0.023$). There were no significant differences in bites, tail rattling, or boxing between the empty implant group and the intact group or control group (all p's > 0.092). Although non-significant, the empty implant group exhibited more bites ($U = 50.00$, $p = .109$) and rattling ($U = 62.00$, $p = .328$) than the control group (which received a T-implant).

Discussion

The correlational experiment and the hormone manipulation experimental data in conjunction suggest that estrogen moderates aggressive behavior in domestic CD-1 mice through a discrete pathway in areas of the hypothalamus and limbic system, specifically the vBNST, LS, and AH. Fadrozole decreased aggression and aggressive behavior was positively correlated with the number of ERα–ir cells in the vBNST, LS, and AH but not in the MPOA or VMH. These results coincide with studies of other species, including the Syrian hamster in which the vBNST and LS were found to be activated in both agonistic and mating interactions while the AH was only activated in agonistic behavior and the MPOA only in mating behavior (Kollack-Walker & Newman, 1995). The results support the hypothesis that a specific subset of regions of the hypothalamus and limbic system act to modulate aggressive behavior.

Previous research has indicated that estrogen increases the likelihood of aggressive behavior in various species of domestic mice (Simon and Whalen, 1986). Extending earlier findings, these experiments show that estrogen receptor immunoreactivity in the brain is positively correlated with aggressive behavior and further, suggest a possible pathway of

modulation by revealing that the relationship is limited to the vBNST, LS, and AH. Most of the regions of the hypothalamus and limbic system examined in this study are believed to play a role in both aggressive and sexual interactions. However, it has been suggested that the AH is minimally activated in sexual contexts, and that the MPOA is minimally activated in aggressive contexts (Newman, 1999 and Kollack-Walker, 1995). In Syrian hamsters (*Mesocricetus auratus*), the AH has been implicated as a brain region in the neural network which regulates offensive aggression (Delville et al., 2000).

In the hormone manipulation experiment, estrogen was shown to increase aggressive behavior in CD-1 mice. The fadrozole group displayed significantly fewer bites and bouts of boxing than the control group which was treated with saline. The data were analyzed with non-parametric tests due to the large variability in aggressive behavior. This variability suggests that estrogen is not the only factor modulating aggression. It is clear that there is at least one other factor influencing the expression of aggressive behavior, but most likely there are many more such factors. As evidenced by the empty implant group, this factor is not likely to be plasma testosterone concentrations. This group was castrated and received an empty implant (as opposed to a T-implant) and was numerically more aggressive than the control group (which received the T-implant) on measures of both bites and tail rattling. This trend was not significant, but it suggests that castration alone does not reduce aggressive behavior. This result has been found in multiple previous studies (Demas et al., 1999 and Trainor and Marler, 2001).

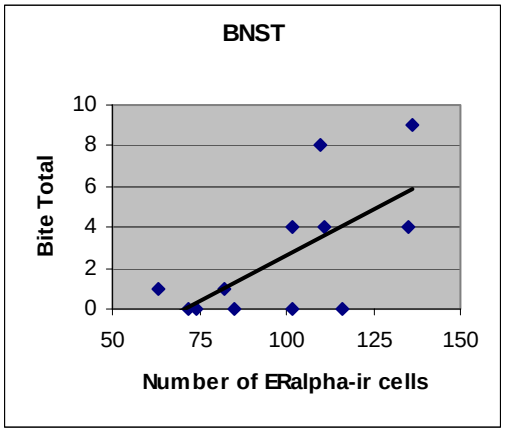
Estrogen had been established previously as a modulator of aggression, but its area of action in the brain was not entirely clear. These two experiments suggest a possible pathway in the brain in which estrogens work to influence aggressive behavior. In summary, individual differences in ERα–ir in the vBNST, LS, and AH were positively correlated with aggressive

behavior. Conversely, ERα–ir was not correlated with aggressive behavior in other brain regions including the MPOA and VMH. This suggests that the effects of estrogen on aggression may occur primarily in these regions, but further research must be conducted to test this hypothesis directly.

Figure Caption

Figure 1. Correlations between ERα immunoreactivity and total number of bites in the (a) vBNST, (b) LS, (c) AH, (d) MPOA, and (e) VMH.

(a)



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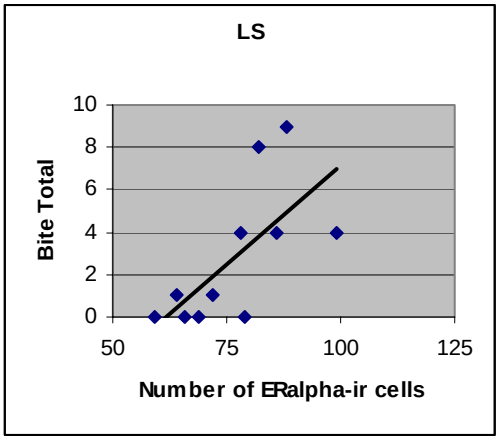

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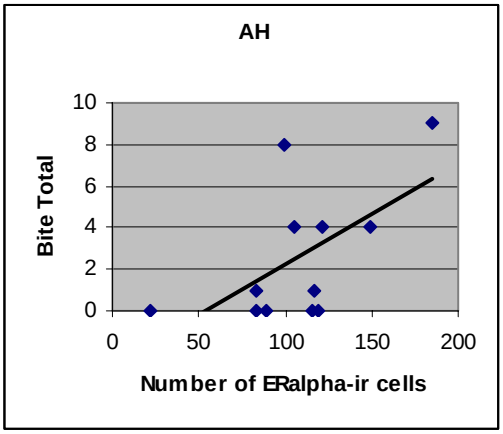

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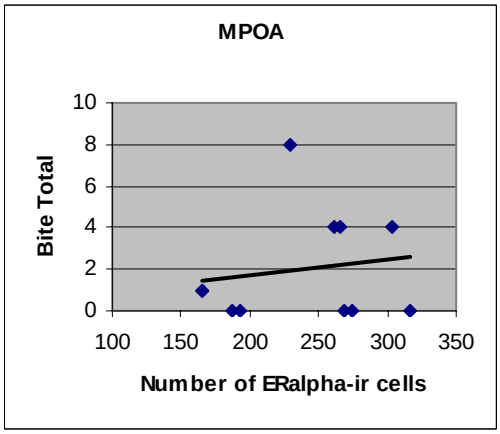


(e)

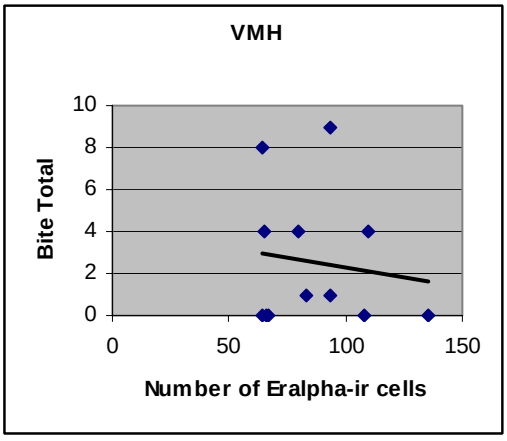
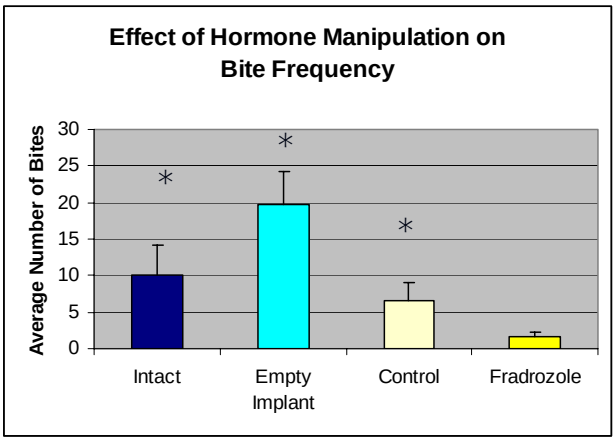
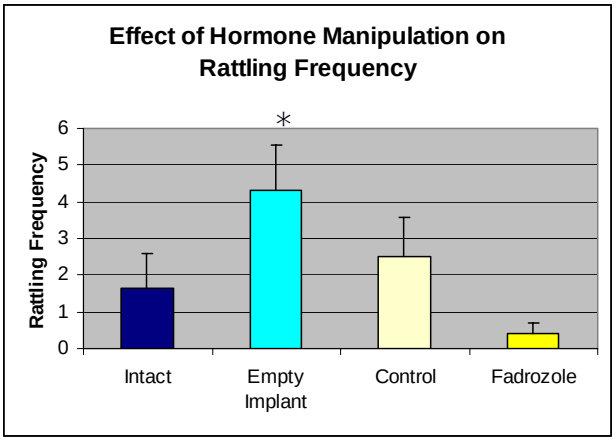


Figure 2. Effect of hormone manipulation on aggressive behavior. Bars represent the mean ± standard error for the intact group (n = 11), empty implant group (n = 16), control group (n = 10), and fadrozole group (n = 12): (a) Bite frequency, (b) Rattle frequency, and (c) Boxing frequency. * Indicates significantly different from the fadrozole goup.

(a)



(b)



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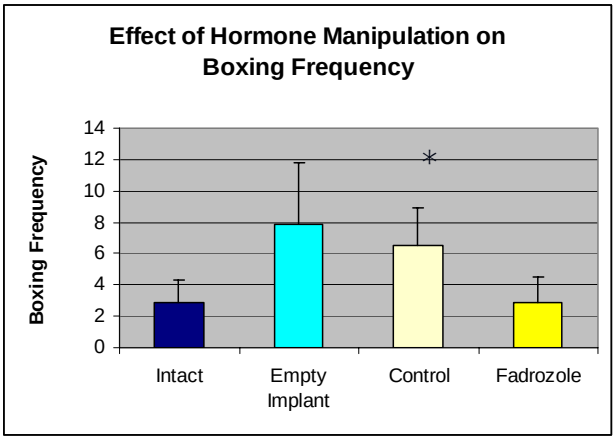

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